Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial.

Anna Boronat[1,2], Julian Mateus[1], Natalia Soldevila-Domenech[1,2], Mercè Guerra[2], Jose Rodríguez-Morató[2], Carlota Varon[3], Daniel Muñoz[4,5], Francina Barbosa[6], Juan Carlos Morales[7], Andreas Gaedigk[8], Klaus Langohr[1,9], Maria-Isabel Covas[4,10], Clara Pérez-Mañá[11,12], Montserrat Fitó[4,5], Rachel F. Tyndale[13], Rafael de la Torre[1,2,5*].

[1]Integrative Pharmacology and Systems Neuroscience Research Group, Neurosciences Research Program, IMIM-Institut Hospital del Mar d'Investigacions Mèdiques, Dr. Aiguader 88, 08003 Barcelona, Spain

[2]Department of Experimental and Health Sciences, Universitat Pompeu Fabra (CEXS-UPF), Dr. Aiguader 80, 08003 Barcelona, Spain

3 Department of Pharmacy, Vall d'Hebron Barcelona Hospital Campus, Passeig de Vall d'Hebron 119-129, 08035, Barcelona, Spain

[4]Cardiovascular Risk and Nutrition Research Group, IMIM (Hospital del Mar Research Institute), Dr. Aiguader 88, 08003, Barcelona, Spain.

[5]CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN, CB06/03/028), Monforte de Lemos 3-5, 28029 Madrid

[6]CAP Barceloneta, Parc Sanitari Rovira Virgili, Passeig Marítim, 25 08003, Barcelona, Spain

[7]Department of Biochemistry and Molecular Pharmacology, Instituto de Parasitología y Biomedicina López Neyra, CSIC, PTS Granada, Avda. del Conocimiento, 17, 18016 Armilla, Granada, Spain.

[8] Children's Mercy Kansas City, Division of Clinical Pharmacology, Toxicology and Therapeutic Innovation, and University of Missouri-Kansas City School of Medicine, Kansas City, Missouri.

[9] Department of Statistics and Operations Research, Polytechnic University of Catalonia, Barcelona, Spain.

[10] NUPROAS Handesbolag (NUPROAS HB). Nacka, Sweden

[11]School of Medicine, Universitat Autònoma de Barcelona, Bellaterra, Spain.

[12]Hospital Universitari Germans Trias i Pujol (IGTP), Badalona, Spain.

[13]Campbell Family Mental Health Research Institute, Centre for Addiction and Mental Health, Departments of Pharmacology & Toxicology, and Psychiatry, University of Toronto; 1 King's College Circle, Toronto, ON M5S 1A8, Canada

Correspondence: Rafael de la Torre, PharmD, PhD. Integrative Pharmacology and Systems Neuroscience Research Group, IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain. Tel: +34 933160484; Fax: +34 933160467; E-mail: rtorre@imim.es

HIGHLIGHTS

- Tyrosol (Tyr) is converted into hydroxytyrosol (OHTyr) *in vivo* in humans
- P450 enzymes *CYP2A6* and *CYP2D6* mediate this bioconversion
- Ingestion of Tyr and its conversion into OHTyr improve endothelial function
- Tyr and its OHTyr conversion improve HDL cholesterol, vasodilatory, and inflammatory markers
- A new mechanism by which foods rich in Tyr could be a source of OHTyr is described

Abstract

Introduction: The simple phenol hydroxytyrosol (OHTyr) has been associated with the beneficial health effects of extra virgin olive oil. Pre-clinical studies have identified Tyr hydroxylation, mediated by cytochrome P450 isoforms *CYP2A6* and *CYP2D6,* as an additional source of OHTyr.

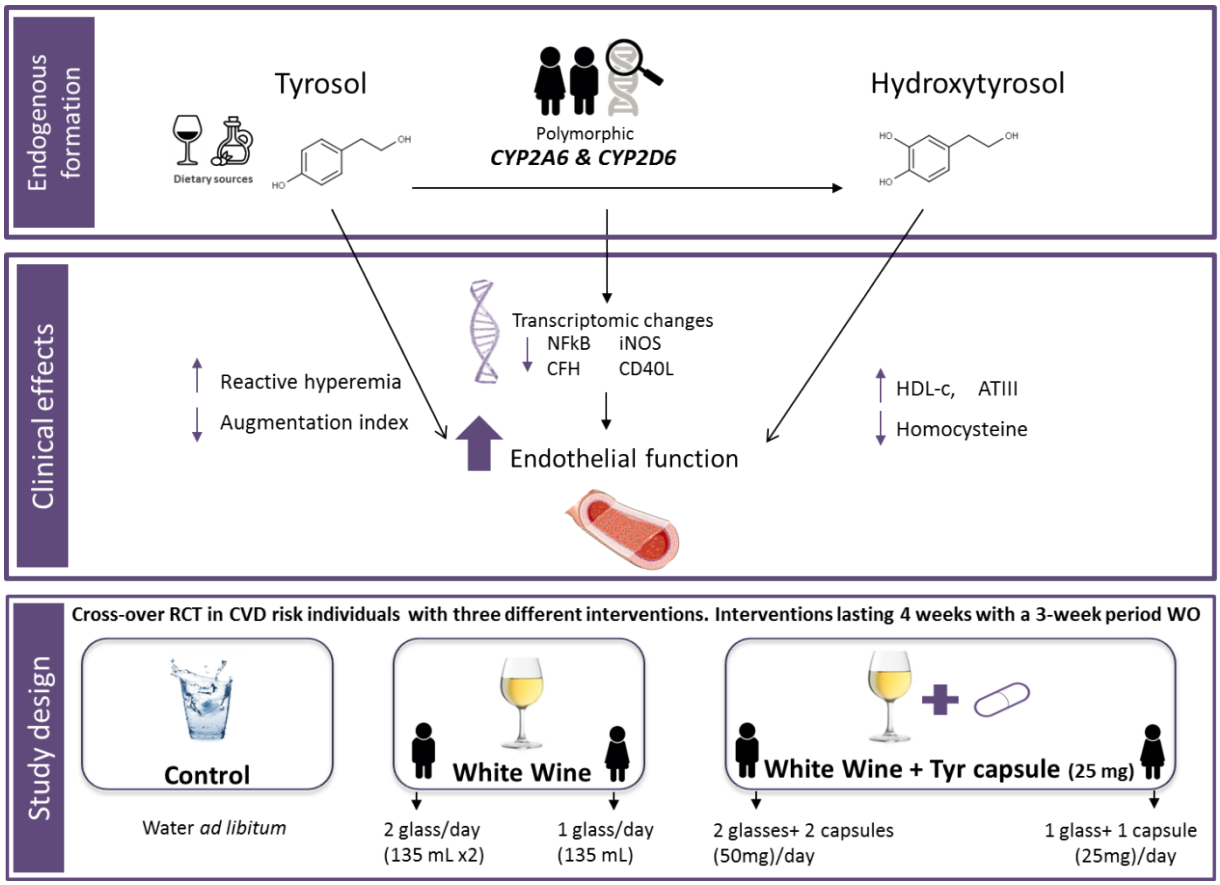
Aim: We aimed to (i) confirm Tyr to OHTyr bioconversion *in vivo* in humans, (ii) to assess the cardiovascular benefits of this bioconversion, and (iii) determine their interaction with a polygenic activity score (PAS) from *CYP2A6* and *CYP2D6* genotypes.

Methods: Randomized, crossover**,** controlled study. Individuals at cardiovascular risk (n=33) received: white wine (WW) (females 1, males 2 standard drinks/day), WW plus Tyr capsules (WW+Tyr) (25mg Tyr capsule, one per WW drink), and water (control) *ad libitum*. Participants were classified by a PAS as low versus normal activity metabolizers.

Results: OHTyr recovery following WW+Tyr was higher than after other interventions ($P<0.05$). Low PAS individuals had lower OHTyr/Tyr ratios compared to individuals with normal PAS. WW+Tyr improved endothelial function, increased plasma HDL-cholesterol and antithrombin IIII, and decreased plasma homocysteine, endothelin 1, and *CD40L, P65/RELA*, and *CFH* gene expression in peripheral blood mononuclear cells ($p<0.05$). Combining Tyr capsule(s) with WW abolished the increase in *iNOS, eNOS, VEGFA,* and *CHF* expressions promoted by WW ($p<0.05$).

Conclusions: Tyr, and its partial biotransformation into OHTyr, promoted cardiovascular health-related benefits in humans after dietary doses of Tyr. The study design allowed the health effects of individual phenols to be singled out from the dietary matrix in which they are naturally found.

Graphical Abstract



Keywords: CYP2A6, CYP2D6, Endothelial function, Hydroxytyrosol, Tyrosol, Wine

Abbreviations

AHA, American Heart Association

AI, augmentation index

ATIII, antithrombin III

CD40L, CD40 ligand

CFH, complement factor H

CVD, cardiovascular disease

CYP2A6, cytochrome P450 2A6 isoform

CYP2D6, cytochrome P450 2D6 isoform

EFSA, European Food Safety Authority

ET1, endothelin 1

Hcy, homocysteine

HDL-c, high density lipoproteins cholesterol

HVAL, homovanillic alcohol

LA, low activity metabolizers

NA, normal activity metabolizers

eNOS, endothelial nitric oxide synthase

iNOS, inducible nitric oxide synthase

OHTyr, hydroxytyrosol

PAS, polygenic activity score

PBMC, peripheral blood mononuclear cells

p65/RELA, transcription factor p65

RHI, reactive hyperemia index

Tyr, tyrosol

VEGFA, vascular endothelial growth factor A

VOO, virgin olive oil

WW, white wine

WW+Tyr, white wine plus tyrosol capsules

1.Introduction

Hydroxytyrosol (OHTyr), a prototypic virgin olive oil (VOO) phenolic compound, has strong antioxidant activity. Pre-clinical studies have attributed to OHTyr anti-inflammatory, anti-proliferative, pro-apoptotic, anti-microbial, and neuroprotective properties [1]. Several clinical trials have shown that OHTyr-rich VOO, alone or in combination with a Mediterranean dietary (MeDiet) pattern, improves lipid profile, protects against lipid oxidation, and prevents primary cardiovascular disease (CVD) outcomes [2,3]. In the EUROLIVE study, [2] olive oil phenols were shown to prevent LDL oxidation in vivo in humans. In November 2011, the European Food Safety Authority (EFSA) released a claim concerning the benefits of the daily ingestion of 5 mg or more of OHTyr and its derivates, e.g. tyrosol (Tyr), from olive oil on protecting low density lipoproteins (LDL) from oxidation [4].

OHTyr direct antioxidant activity is attributed to its ortho-diphenol moiety, capable of scavenging free radicals in vitro. Because free radical activities should play a central role in the prevention of LDL oxidation by olive oil phenols, further studies have investigated their radical scavenging capacities. OHTyr and other olive oil phenols were tested in the AAPH(2,2-azobis(2-amidinopropane) dihydrochloride))-induced oxidation model. The inhibition of lipids oxidation was assumed to be related to a reduction of the AAPH-derived initiating radicals. Olive oil phenols were shown to be efficient scavengers of hydrophilic peroxyl radicals with a long-lasting antioxidant effect owing to the residual activity of some of their oxidation products (oligomer formation with the addition of water molecules) [5]. The radical-scavenging capacities (hydrogen donation abilities) of Tyr and OHTyr were also investigated using the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay. The 24-h kinetics of DPPH radical scavenging

(percentage of remaining DPPH) showed that OHTyr appeared to be a stronger antiradical scavenger than Trolox whereas TYR was found to be practically inactive [6].

Nevertheless, equivalent antioxidant effects *in vivo* are limited by bioavailability, concentration and location of the antioxidant in cells. However free radical scavenging cannot be discounted in the gut where concentrations of OHTyr after food intake are sufficiently high. Therefore, Forman et al (2014) stated that the most likely effect of the so-called nutritional antioxidants would be mediated by a process called para-hormesis; by inducing the endogenous antioxidant mechanism [7]. Polyphenols and polyphenol rich food, such as rich-phenolics olive oil, modulate paraoxonase 1(PON1) activity and expression [8,9]. Also polyphenols alter the expression of genes underlying atherosclerosis- and cancer-related pathways [10], and olive oil polyphenols enhance the expression of cholesterol efflux related genes *in vivo* in humans [11].

Both wine and VOO, two characteristic components of the MeDiet, are sources of OHTyr and Tyr [4,12]. Within the framework of the PREDIMED (Prevención con Dieta Mediterránea) Study, we previously reported 1) a direct dose-dependent association between OHTyr urinary concentrations and wine or alcohol consumption in individuals at high risk of cardiovascular diseases (CVD) [13], and 2) an independent association between high urinary concentrations of 3-O-methyl-hydroxytyrosol (HVAL), a OHTyr biological metabolite, and a lower risk of CVD and total mortality in CVD risk individuals [14]. In addition, in previous work [15–17], we have shown that alcohol and red wine could promote *di novo* OHTyr generation *in vivo* in humans. We identified alterations in dopamine and tyramine oxidative metabolism, as well as an increased Tyr bioavailability due to ethanol, as mechanisms involved [17]. Results from our animal studies suggested biotransformation of Tyr to OHTyr as another related mechanism. In *in vitro* enzymatic

studies in human liver samples identified the genetically polymorphic isoenzymes of cytochrome P450, *CYP2A6*, and *CYP2D6*, as involved in the biotransformation of Tyr into OHTyr [18]. *CYP2A6* and *CYP2D6* enzymatic activities have extensive inter-individual and world population variation, primarily due to genetic variation. Although the health benefits of OHTyr ingestion *in vivo* in humans have been investigated [19], those of Tyr, per se, are at present unknown.

In this context, we performed a randomized, crossover, controlled trial in cardiovascular risk individuals with white wine, white wine plus Tyr capsules, and water acting as control. Our primary hypothesis was that the Tyr ingested would be bio-transformed into OHTyr and together they would promote benefits on cardiovascular risk in humans. Our secondary hypothesis was that these processes would be modulated by the different genotypic profiles for *CYP2A6* and *CYP2D6.*

2. Experimental Section

2.1. Wine and Capsule Characteristics

The white wine used in the experiments (12.5 % Alc. Vol., Bach Viña Extrísimo Blanco Seco 2016) was provided by the Codorniu Raventós Group (Barcelona, Spain). Tyr content (10.4 ± 0.6 mg/L) and that of OHTyr (1.3 ± 0.1 mg/L) was quantified by liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS). Gelatin white opaque capsules containing 25 mg Tyr/unit were produced by the manufacturing department of the Jordi Cabezas Pharmacy (Barcelona, Spain).

2.2. Study Population

Recruitment was carried out through a volunteer center database, primary healthcare centers, and word of mouth, starting on January 2016 and finishing on

December 2017. Inclusion criteria included being at high risk for coronary heart disease (CHD) with 3 or more risk factors. Inclusion and exclusion criteria are described in Data in Brief [20]. Subjects were interviewed to exclude concomitant medical conditions, and they underwent a general physical examination, laboratory tests, and a 12-lead ECG. The study was conducted in accordance with the Helsinki Declaration and approved by the local Ethical Committee (CEIm-Parc de Salut Mar) and registered in ClinicalTrials.gov database (NCT02783989). Written informed consent to participate was obtained prior to any study-related procedure.

2.3. Study Design

A randomized, crossover, controlled study was performed at the Clinical Research Unit of the Hospital del Mar Medical Research Institute (IMIM, Barcelona, Spain)**.** Participants were randomly assigned to one of six orders of administration to receive: water (control), white wine (WW), and WW plus tyrosol capsules (WW+Tyr) [20]. Participants were randomly allocated to the three types of intervention, through a computerized block-randomization method for sequence generation, by an independent statistician. Intervention periods lasted for four weeks preceded by three week wash-out periods in which participants were requested to avoid additional sources of alcohol, and to follow a low-phenol content diet [20]. In the WW periods, women ingested one glass of WW (135 mL, 13.5 g of alcohol, 1.4 mg of Tyr, and 0.2 mg of OHTyr), whereas males ingested two glasses of WW (270 mL, 27 g of alcohol, 2.8 mg of Tyr, and 0.4 mg of OHTyr). WW was ingested during meals; women drank one glass at lunch time while men drank one glass at lunch time and another glass at dinner time. In the WW+Tyr periods, participants ingested the same amount of WW as described above but in combination with capsules containing 25 mg Tyr: 2 for men (50 mg), and 1 (25 mg) for

women. During the control intervention subjects were only allowed to drink water (no alcohol, wine, or supplemented Tyr or OHTyr). The reason for different WW doses for men and women was in order to follow the American Heart Association (AHA) guidelines, which limit alcohol consumption to one drink per day in women and two in men, preferably taken at meals [21]. The doses of wine administered are within those recommended by the AHA, and that of Tyr, 25 to 50 mg/day, within the range of its maximal content per liter in red and fortified wines (e.g. sherry) (http://phenol-explorer.eu/contents/polyphenol/673).

A 24 h food recall was performed to assess dietary intake before and after each intervention period. Physical activity was recorded at the beginning and end of the study and assessed by the Minnesota Leisure Time Physical Activity Questionnaire, validated for the Spanish population [22]. A general physical examination, and routine urine, blood chemical and hematological analyses, were performed at the beginning and end of the study. Blood and 24h-urine samples were collected at fasting state before and after each intervention period. Blood was collected into 10 mL tubes containing EDTA and centrifuged (1700g, 15 min, 4 °C), and plasma and buffy coat samples were then isolated. Peripheral blood mononuclear cells (PBMC) were isolated using a Vacutainer Cell Preparation Tube (CPT™) and kept for RNA extraction. Plasma, urine, and PBMC samples were frozen at -80 °C until analysis. Genomic DNA isolation from buffy coat was performed with QIAamp DNA Blood Midi Kit (Qiagen, Dusseldorf, Germany).

2.4 Compliance biomarkers

The urinary concentrations of Tyr and OHTyr metabolites were determined from samples collected before and after each intervention following a validated methodology [20]. Total Tyr and OHTyr were obtained from the sum of their metabolites. 24 h-Urinary

concentrations of ethyl glucuronide, measured by HPLC/MS/MS, were assessed as a marker of alcohol consumption [23] .

2.5. Genotyping

Volunteers were genotyped for different allelic variants of *CYP2A6* and *CYP2D6* using TaqMan genotyping assays (Applied Biosystems, Foster City, CA, USA) [20]. Tested allelic variants were categorized into: non-functional (*CYP2A6 *2, *4; CYP2D6 *4,*5*); decreased function (*CYP2A6 *9,*12; CYP2D6 *9,*10,*11*); functional (*CYP2A6 *1; CYP2D6 *1,*2,*35*); and increased function (*CYP2A6 *1xN*; *CYP2D6 *1xN,*2xN,*35xN*). *CYP2D6* gene duplications were further confirmed by… A score of 0, 0.5, 1 or 2 was assigned for the presence of each allele [20]. For each enzyme, an activity score was assigned to each volunteer according to the alleles identified, based on the method described by Gaedigk et al [24]. A pooled polygenic activity score (PAS) was calculated by adding the two enzyme activity scores together. According to their PAS, individuals were placed into three groups of phenotype activity: low activity (LA), normal activity (NA), and rapid activity (RA) groups.

2.6. Primary Outcomes

Endothelial function was assessed before and after interventions by monitoring endothelium-mediated changes (reactive hyperemia index, RHI) in the digital pulse waveform, known as the Peripheral Arterial Tone (PAT) signal (EndoPAT 2000; Itamar Medical Inc., Caesarea, Israel). The hyperemic reactivity index measured by EndoPAT 2000 has been shown to predict cardiovascular disease [25]. EndoPAT 2000 also provides the augmentation index (AI), a measurement of arterial stiffness via pulse-wave analysis. Measurements were performed by a trained professional with the participants in resting

supine conditions, in a quiet room, at a constant temperature, after 10 minutes of stabilization [20].

2.7. Secondary Outcomes

2.7.1. Cardiovascular Risk Biomarkers

All analyses were carried out before and after interventions, and samples from the same individual were analyzed in the same analytical run. Plasma glucose, triglycerides, total and HDL cholesterol (HDL-c), antithrombin III (ATIII), and dimer D were measured by automated enzymatic methods. LDL cholesterol was calculated by the Friedewald formula whenever triglycerides were inferior to 300 mg/dL. Serum high-sensitivity C reactive protein (hs-CRP) was determined by immunoturbidimetry (Horiba, Montpellier, France). Homocysteine (Hcy) in plasma was measured by gas chromatography-mass spectrometry (GC-MS) after liquid-liquid extraction. Nitrites and nitrates in plasma were determined by a colorimetric kit (Cayman Chemical, Michigan, USA). Endothelin 1 (ET1) and plasminogen activator inhibitor-1 (PAI-1) were measured in plasma by ELISA (Invitrogen, California, USA; and Affymetrix, California, USA, respectively).

2.7.2. Gene Expression

On the basis of their relationship with endothelial health and atherosclerosis, and the available data of gene expression response after VOO ingestion [11,26], several candidate genes were selected. Gene expression was measured, before and after interventions, by a real-time polymerase chain reaction (See in Boronat et al., 2019 [20]).

2.8. Sample Size and Power Analyses

A total sample of 32 participants would allow at least 80% power to detect a statistically significant difference among groups of 0.205 units in the RHI measurement,

assuming a dropout rate of 5% and type I error of 0.005 (2-sided). The standard deviation of the measurement was assumed 0.4 [25].

2.9. Statistical Analyses

Normality of continuous variables was assessed by normal probability plots and data were log transformed when required. Intra-treatment comparisons were assessed by Student's t test for paired samples. Comparisons among treatments were made by an ANOVA for repeated measures adjusted for age, gender, smoking, AAS medication, and baseline concentrations. In the case of lipids an additional adjustment for LDL cholesterol values at the beginning of the study was performed. A general linear model was used to assess linear and quadratic trends. For the post-hoc pairwise comparison, the Tuckey test was used. Statistical analyses were performed with R (R Foundation for Statistical Computing, Vienna, Austria), version 3.0.2., and R package multcomp. Significance was defined as $p < 0.05$.

3.Results

3.1. Study Participants

From the 192 subjects assessed for eligibility, 157 were excluded [20]**.** The remaining 33 participants (21 men and 12 women) were randomly allocated to interventions, and 32 participants completed the study (**Figure 1**). Baseline characteristics of participants are described elsewhere [20]**.** Briefly, participants' mean age was 65.3 ± 6.2 years with a mean BMI of 32.6 ± 4.2 kg/m^2. Main clinical features were overweight/obesity (97.0%), hypertension (84.8%), and dyslipidemia (75.6%). No differences in baseline characteristics were observed among the sequence of administration groups with the exception of LDL cholesterol which was higher (157 ± 24

mg/dL) in sequence 3 than in sequence 5 (98 ± 32 mg/dL). Thus, lipid variables were adjusted by LDL cholesterol in the statistical analyses. No changes in the level of physical activity were observed from the beginning to the end of the study in any group. No dietary differences were observed among interventions [20]**.** On the basis of the polygenic activity score (PAS), 11 individuals were categorized as low (LA, PAS range: 1-2.5), 19 as normal (NA, PAS range: 3-4), and 2 as rapid (RA, PAS range = 5) activity metabolizers. Due to their low number, RAs were excluded for analyses. Age and gender were equally distributed among the three groups.

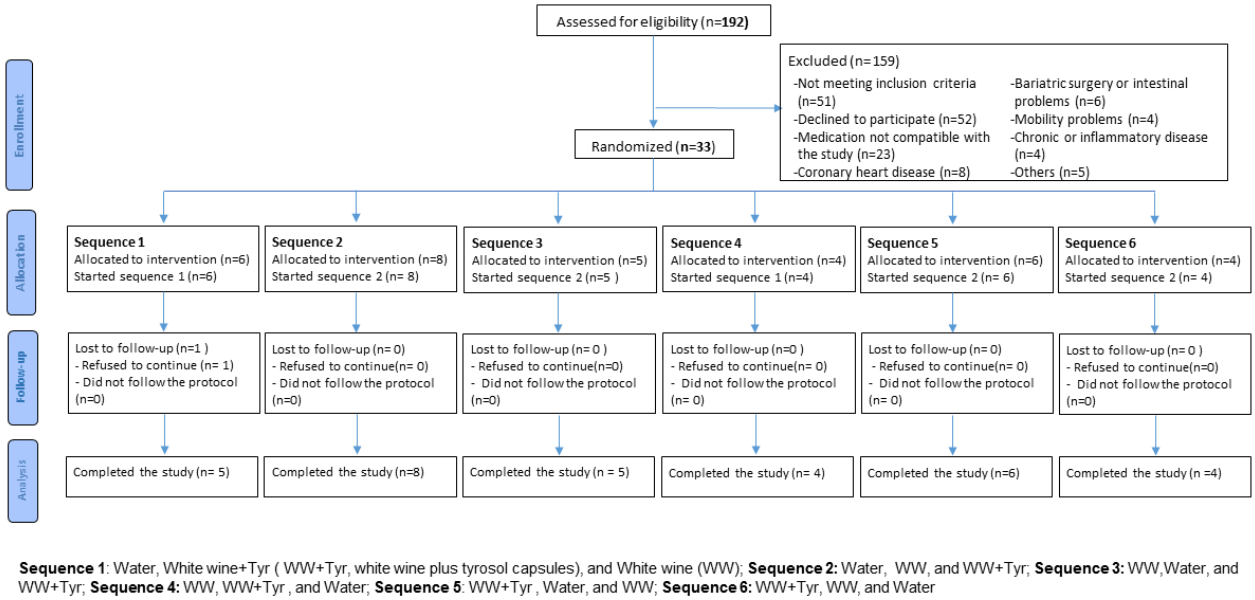


Figure 1. Flow chart of the study

3.2. Compliance Biomarkers and Conversion of Tyrosol in Hydroxytyrosol

Changes in the 24h urinary recoveries of total Tyr and OHTyr in each treatment group are shown in **Figure 2**. Urinary Tyr and OHTyr increased in a dose-related manner with increasing Tyr content of the beverage administered, (p<0.001 and p=0.042, respectively for linear trend). Baseline values of ethyl-glucuronide, and those after water

treatment, were undetectable (i.e. below 4.5 nmol/mL based on assay detection limits). Ethyl-glucuronide urinary total excretion after WW intervention was 1.61 ± 1.11 µmol, and 2.18 ± 1.64 µmol following WW+Tyr.

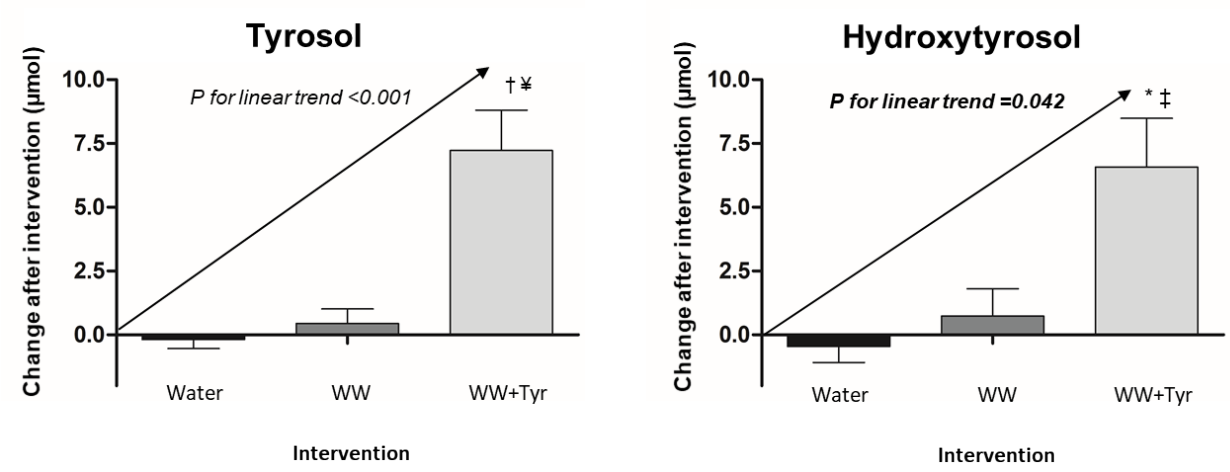


Figure 2. Changes (mean± SEM) in 24h-urinary recovery of tyrosol and hydroxytyrosol metabolites after interventions. Total tyrosol accounted and total OHTyr correspond to the molar sum of their respective quantified metabolites. WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; * p<0.05, † p <0.001 versus water; ‡ p <0.05, ¥, p <0.001 versus WW

3.3 Interaction between PAS, Tyr and OHTyr metabolism

Figure 3 shows Tyr and OHTyr recoveries following WW+Tyr according to the PAS categories. Comparisons were adjusted to the administered dose of Tyr. The LA group presented higher recoveries of urinary total Tyr compared to NA one (p =0.021). Conversely, OHTyr metabolite recovery was higher in NA compared to LA (p=0.115). As expected OHTyr/Tyr ratio were higher in NA compared to LA (p =0.025).

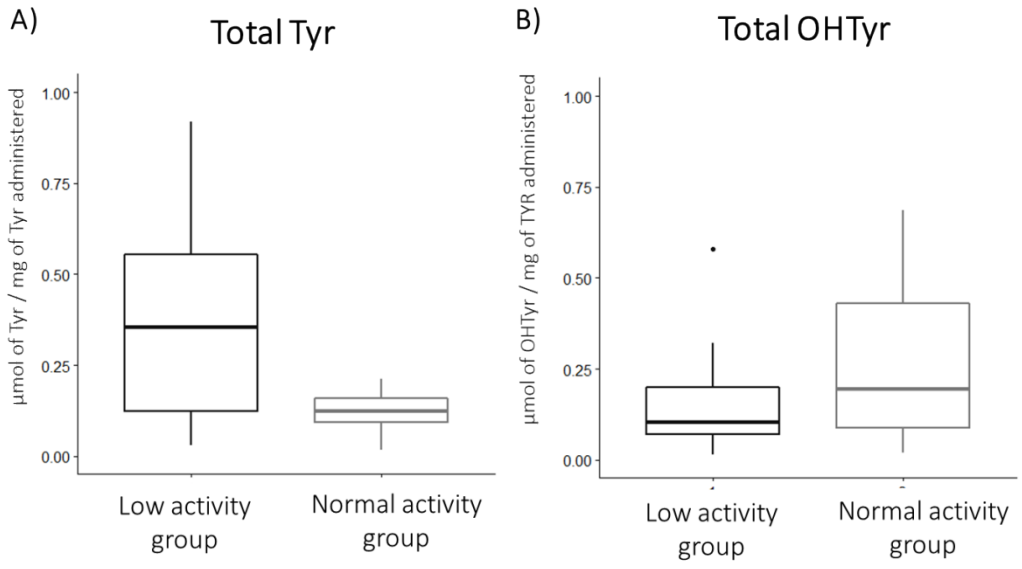


Figure 3. Phenotype interaction with Tyr and OHTyr metabolites following WW+Tyr: recovery of Tyr (A), recovery of OHTyr (B) standardized by Tyr dose administered. Data expressed as median and percentile 10-90th, Between-group comparison: * $p< 0.05$, ** $p< 0.01$.

Figure 4 shows the positive correlation between PAS and OHTyr/Tyr ratio (r= 0.448; p=0.011). These associations were only found using the PAS including both enzymes, and not when analyzed alone for *CYP2A6* or for *CYP2D6* genotype activity scores separately.

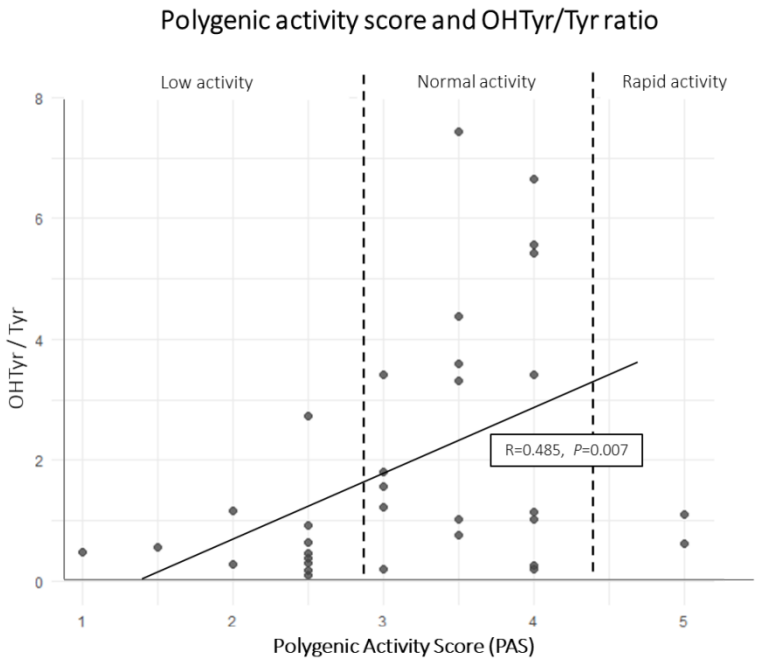


Figure 4. Correlation between the polygenic activity score and the OHTyr/Tyr ratio. Broken lines indicate separation of low, normal and rapid activity groups. The determination coefficient has been assessed excluding individuals from the rapid activity group.

3.4. Primary Outcomes

Table 1 shows the changes in the reactive hyperemia index (RHI) and augmentation index (AI) after each intervention. One volunteer had skin petechiae the first time the ENDOPAT test was performed and endothelial function was not measured in this subject, thus only 31 participants were assessed. RHI increased after the WW+Tyr intervention compared to its baseline (p=0.048*)*, remaining significant only in men (p=0.041) when analyses were stratified by gender. When assessing the PAS-genotype interaction, RHI increased after the WW+Tyr intervention compared to its baseline (p=0.020) only among the LA group, the increase was significant *versus* changes in the WW intervention (p=0.040). AI decreased significantly after WW+Tyr compared to changes in control water intervention (p=0.047) but was not significant when stratified

by gender. When assessing the PAS-genotype interaction, the decrease in AI was significant *versus* changes in the control group in the case of the LA group (p=0.007).

Table 1. Changes in endothelial function after interventions

	Intervention				
	Control	WW	WW+Tyr	*p* value for WW+Tyr	
				vs Control	vs WW
Reactive hyperemia index (AU)					
All participants	0.04 ± 0.40	0.03 ± 0.49	0.17 ± 0.47*	0.521	0.309
Women	0.03 ± 0.54	- 0.08 ± 0.75	0.14 ± 0.35	0.990	0.725
Men	0.06 ± 0.31	0.10 ± 0.22	0.24 ± 0.49*	0.147	0.196
Genotype Interaction					
LA	- 0.04 ± 0.43	- 0.13 ± 0.55	0.33 ± 0.39*	0.252	0.040
NA	0.07 ± 0.39	0.16 ± 0.46	0.07 ± 0.52	0.955	0.987
Augmentation	Index (AU)				
All participants	2.54 ± 10.28	-2.19 ± 11.12	-3.78 ± 17.23	0.047	0.821
Women	5.02 ± 11.47	-4.27 ± 8.64	-2.35 ± 18.60	0.282	0.972
Men	1.07 ± 9.63	-1.22 ± 12.81	-5.18 ± 1715	0.231	0.639
Genotype Interaction					
LA	1.24 ± 6,97	-0.80 ± 8.24	-4.98 ± 15.49	0.007	0.265
NA	2304 ± 11.57	-1.10 ± 9.22	-3.91 ± 10.65	0.436	0.990

Results are expressed as mean ± SD (n=31). AU, arbitrary units; WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex, smoking habits, acetylsalicylic acid consumption, and baseline levels. *$p < 0.05$ versus its baseline; p value, significance for inter-intervention comparisons.

3.5. Secondary Outcomes

3.5.1. Glucose, Lipids, and Inflammatory Biomarkers

Table 2 shows the changes in HDL-c after each intervention. A significant increase from their respective baselines was observed after the WW (p=0.027) and WW+Tyr (p<0.001) interventions, the latter remaining in men when analyses were stratified by gender (p<0.001). The increase in HDL-c after WW+Tyr was significant *versus* changes in the control group (p=0.025), and this significance remained in males (p< 0.001 *vs* control and p= 0.027 *vs* WW) and in the NA group (p=0.014 *vs* control) when analyses were stratified by gender or by PAS genotype, respectively. HDL-c increased in a dose-dependent manner with the content of alcohol plus Tyr administered in all populations (p=0.027 for linear trend), for men only (p=0.001 for linear trend), and with a borderline significance within the NA group (p=0.082) [20]**.** No differences by intervention group, gender, or PAS genotype were observed in total and LDL cholesterol, triglycerides, hsCRP, or glucose [20].

Table 2. Changes in HDL cholesterol (mg/dL) after interventions

	Interventions				
			WW+Tyr	*p* value for WW+Tyr	
	Control	WW	Tyr	vs Control	vs WW
Total	-0.11 ± 5.98	1.62 ± 5.06*	3.11 ± 3.96†	0.025	0.417
Women	2.61 ± 5.11	3.05 ± 6.65	1.27 ± 3.91	0.794	0.697
Men	-1.74 ± 5.99	0.77 ± 3.75	4.17 ± 3.68†	< 0.001	0.027
Genotype interaction					
LA	0.66 ± 9.86	3.35 ± 7.74	3.44 ± 3.20*	0.802	0.995
NA	-0.38 ± 2.59	1.12 ± 2.29*	2.97 ± 4.70*	0.014	0.242

Results are expressed as mean ± SD (n=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex and smoking habits, LDL cholesterol at the beginning of the study,

and baseline levels. *P < 0.05, †P<0.001 versus its baseline. *P* value, significance for inter-intervention comparisons.

3.5.2. Vasodilation related markers

Table 3 show the changes in Hcy concentrations. Hcy increased after WW (p= 0.049) and decreased after the WW+Tyr intervention (p= 0.041) in the total group. WW+Tyr decreased Hcy in both genders (not significantly). When changes among interventions were compared, the decrease after WW+Tyr was significant versus changes after WW (P=0.028). The latter trend remained in the NA group when stratified by PAS genotype (p=0.095). ET1 concentrations after interventions were: 2.2 ± 0.9, 2.3 ± 1.1 ng/mL, and 2.0 ± 0.8 ng/mL, after water, WW, and WW+Tyr interventions respectively [20]. Concentrations of ET1 after WW+Tyr were significantly lower versus WW intervention (p=0.031). No differences were observed when analyses were stratified by gender. No differences by intervention group, gender, or PAS groups were observed in nitrate and nitrite values.

Table 3. Homocysteine changes (μmol/L) after interventions

	Interventions				
				p value for WW+Tyr	
	Control	WW	WW+Tyr	vs Control	vs WW
All participants	- 0.07 ± 1.11	0.40 ± 1.11*	-0.36 ± 0.97*	0.975	0.028
Women	-0.23 ± 1.20	0.72 ± 1.36	-0.61 ± 1.20	0.675	0.315
Men	0.01 ± 1.11	0.20 ± 0.90	-0.24 ± 0.80	0.826	0.358
Genotype interaction					
LA	0.13 ± 0.58	0.50 ± 1.01	-0.26 ± 0.85	0.973	0.533
NA	-0.19 ± 1.45	0.43 ± 1.20	-0.47 ± 1.06	0.963	0.095

Results are expressed as mean ± SD (n=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex smoking, acetylsalicylic acid consumption, and baseline levels. *p < 0.05 versus its baseline; P value, significance for inter-intervention comparison

3.5.3. Coagulation and fibrinolysis biomarkers

Table 4 shows the changes in ATIII after each intervention. ATIII increased after WW+Tyr compared to its baseline (p=0.005), the increase was significant versus changes after control (p=0.044) and WW (p=0.005) interventions. When the data were stratified by gender, a borderline increase (p=0.060) after WW+Tyr occured in males, the increase was significant versus changes after WW (p=0.002). Concerning PAS groups, after the WW+Tyr intervention, ATIII plasma concentrations increased versus its baseline (p=0.010) in the NA group. No differences by intervention group, gender, or PAS groups were observed in PAI and dimer D plasma concentrations.

Table 4. Changes in antithrombin III (mg/dL) after interventions

	Interventions				
				p value for WW+Tyr	
	Control	WW	WW+Tyr	vs Control	vs WW
All participants	0.000 (-2.97; 0.00)	0.000 (-0.35; 0.17)	0.000* (0.00; 7.35)	0.044	0.005
Women	0.000 (-2.55; 0.00)	0.000 (0.00; 0.07)	0.000 (0.00; 6.62)	0.422	0.491
Men	0.000 (-3.20; 0.67)	0.000 (-5.57; 0.35)	0.700 (0.65; 9.00)	0.205	0.002
Genotype interaction					
LA	1.900 (0.49; 11.4)	0.000 (-9.10; 4.45)	0.700 (-1.60; 2.70)	0.670	0.054
NA	0.000 (-2.98; 0.00)	0.000 (0.00; 0.15)	0.000* (0.00; 3.85)	0.102	0.444

Results are expressed as median (25^{th}-75^{th}percentiles) (n=32). WW, white wine; WW+Tyr, white wine plus tyrosol (Tyr) capsules; LA, low activity group metabolizers; NA, normal activity group metabolizers. ANOVA adjusted by age, sex and smoking habits, and baseline levels. Prior to

statistical analysis, data were transformed using the following formula ln(x+1)). *P < 0.05, †P<0.01 versus its baseline; P value, significance for inter-intervention comparisons.

3.5.4. Gene expression

Figure 5 shows comparisons among percentage of changes in the expression of genes related to cardiovascular risk with significant changes in all populations. Subgroup changes are depicted in Boronat et al., 2019 [20]. *CD40L* expression decreased following WW+Tyr (p<0.001), the decrease was significant versus changes after control (p=0.041) and WW (p=0.003) interventions. When analyses were stratified by gender, *CD40L* decreased versus its baseline after WW+Tyr in both genders (p<0.05), the decrease was significant versus control (p=0.016) and WW (p=0.024) for males. Concerning PAS groups, in the NA group the *CD40L* expression decreased after WW+Tyr versus control (p=0.011) and WW(p=0.0.020) groups. The expression of *P65/RELA* decreased versus changes after WW intervention in all subjects (p=0.048). *CFH* expression increased *versus* its baseline in all subjects and among women (p<0.05) after WW intervention. After the WW+Tyr intervention, there was a decrease versus changes after WW intervention in all subjects (p=0.025). When analyses were stratified by gender, there was a decrease in males versus baseline after the WW+Tyr intervention (p=0.010) which was significant versus control (p= 0.013) and WW (p=0.048). *iNOS* increased after WW intervention (P=0.032), the increase was significant versus the decrease observed after WW+Tyr intervention in the all subjects (p=0.007) and among males (p=0.019). The expression of *eNOS* and *VEGFA* increased after the WW intervention (p=0.034 and p=0.045, respectively), and decreased after the WW+Tyr intervention versus changes after the WW intervention in all subjects (p=0.045). No changes were observed in expression of other genes which were evaluated.

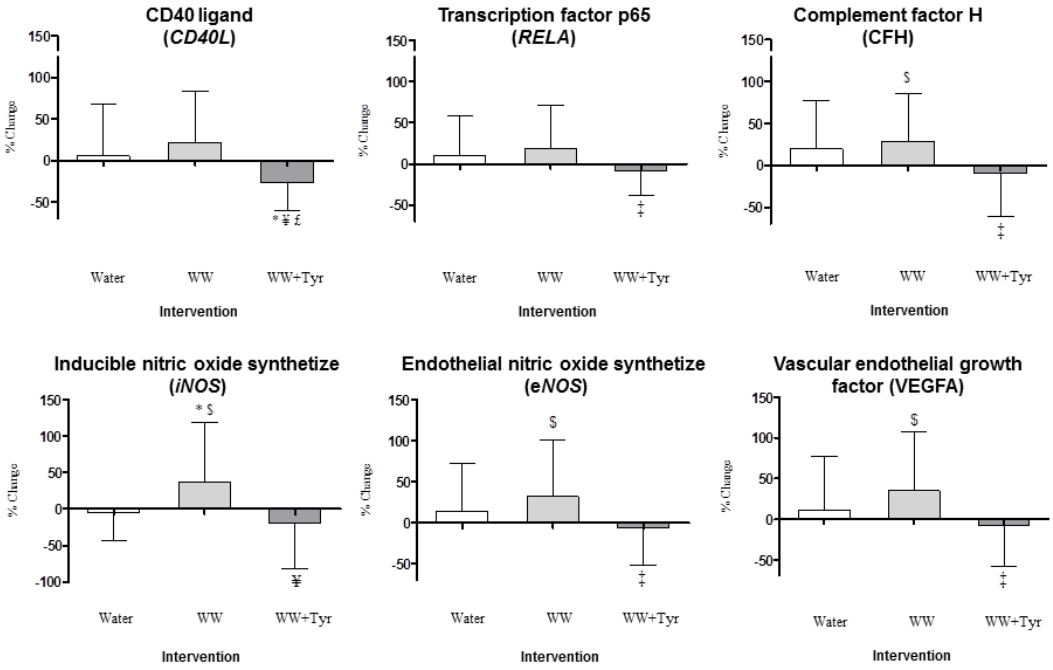


Figure 5. Changes in the expression of cardiovascular related risk genes. WW, white wine; WW+Tyr, white wine plus tyrosol capsules. * $p<0.05$ *versus* water; ‡ $p<0.05$, ¥ $p<0.001$ *versus* white wine; $ $p<0.05$, £ p<0.001 versus its baseline.

4. Discussion

The present study reports occurange of endogenous bioconversion of Tyr into OHTyr in vivo and confirms the *in vivo* modulation of this reaction by variation in CYP2A6 and CYP2D6. Our findings enhance our understanding of the in vivo biological activity of Tyr and OHTyr. This activity goes beyond simple ROS scavenging, to being capable of modulating crucial signaling pathways. This data indicates that both Tyr *per se* and its endogenous conversion into OHTyr promote cardiovascular health benefits in humans. Ingestion of an enriched WW+Tyr improved endothelial function, plasma levels of HDL-c, Hcy, ET1, and ATIIII, and the expression of *CD40L, p65/RELA,* and *CFH* in PBMC. Addition of Tyr to WW abolished the increase in *iNOS, eNOS, VEGFA,* and *CHF*

observed after WW. Tyr seems the preferential phenol involved in benefits on RHI and AI, whereas OHTyr appears to be the leading phenol for explaining HDLc, Hcy, ATII systemic levels, and *CD40L* expression improvements. A schema of the possible interrelationships among these variables is depicted in **Figure 6**, and comparisons among changes after WW and WW+Tyr in Boronat et al., 2019 [20].

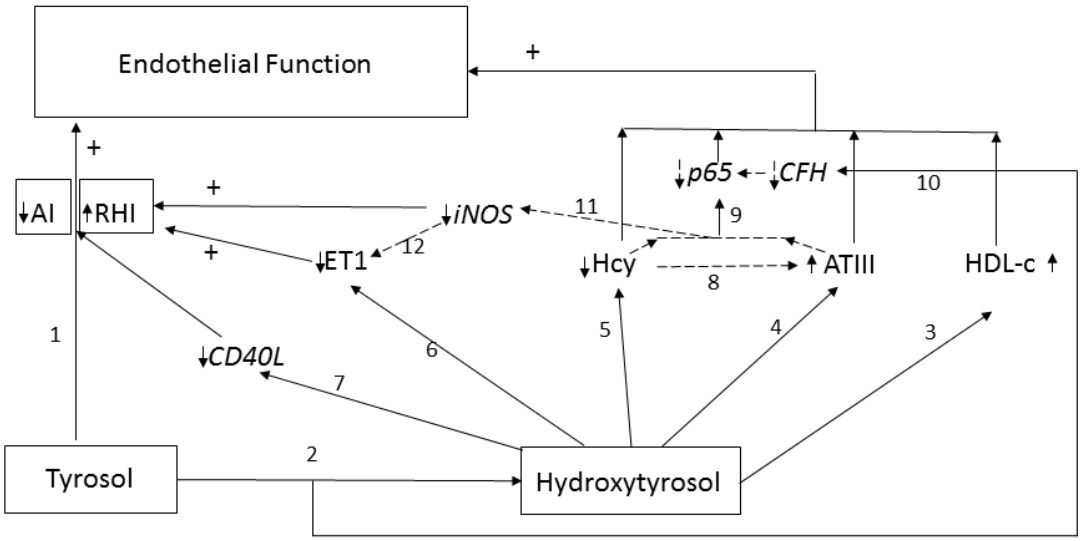


Figure 6. Interrelationship among tyrosol and its biotransformation into hydroxytyrosol with endothelial function and its risk factors. 1, tyrosol seems to be the main phenol involved in the direct improvement of reactive hyperemia (RHI) and arterial stiffness (AI). Its conversion to hydroxytyrosol (2), however, also improves endothelial function through an increase in HDL-c (3) and antithrombin III (ATIII) (4), and a decrease in homocysteine (Hcy) (5), endothelin 1 (ET1) (6), *CD40L* (7), and *p65* (9) expressions. The increase in ATIII could be mediated by the decrease in Hcy (8) and both changes could improve endothelial function through a reduction in *NFkB* (*p65-RElA*) (9), potentiated by the *CFH* decrease (10), and in *iNOS* (11) that in turn contributes to ET1 reduction (12). Full lines depict the study findings, and dashed linesindicate related mechanisms.

In the present report we show a novel biochemical mechanism by which foods rich in Tyr could be a source of OHTyr. OHTyr formation is of relevance since most healthy effects attributed to EVOO in the context of the MeDiet are attributed to the profile of monounsaturated fatty acids and to its content of OHTyr. The relevance of OHTyr in the MeDiet has been further supported with an independent association between high homovanillyl alcohol concentrations, a stable OHTyr metabolite, and a lower risk of CVD and total mortality in elderly individuals [14]. The effects of VOO, however, cannot be directly extrapolated to the effects of pure OHTyr or pure Tyr. In this study, WW was chosen as matrix for performing the clinical studies for two reasons. First, wine alcohol content improves Tyr bioavailability [17]. Secondly, the content of phenolic compounds of WW is the lowest from among grape products (like red or sparkling wine) reducing confounding of interpretation. In this context, the coadministration of Tyr in the form of capsules and its bioconversion to OHTyr can be observed easily over the background of phenolic compounds already present in the white wine administered. In this study, the WW intervention allowed the determination of the OHTyr formation due to the ethanol, via dopamine oxidative metabolism, with the phenolic compounds present in the matrix (mainly Tyr); together estimated to be of 19% over baseline. In contrast, WW+Tyr resulted in a 283% increase compared to baseline of OHTyr recovery. This difference between WW and WW+Tyr interventions can, essentially, be attributed only to Tyr biotransformation to OHTyr.

To the best of our knowledge, there is no data assessing the toxicological effects of high doses of Tyr. Based on OHTyr toxicology, EFSA has established a no-observed adverse effect level (NOAEL) of 50mg/kg/day. Due to the similarity of the molecules, we do not expect any harmful effects from the given dose of Tyr as it was far below the

OHTyr NOAEL. Nonetheless, toxicological studies may need to be performed to ensure Tyr safety [27].

Endothelial dysfunction is a predictor for CVD [25,28]. In our study RHI, a marker for the endothelium-dependent vasodilator function [29], increased by a mean of 0.176 AU after WW+Tyr. This value corresponds to a 0.103 AU when data are on a logarithmic scale (Ln-RHI). A 0.1 increase in Ln-RHI has been associated with a 21% decrease in CVD according a metanalysis that included 1602 individuals [30]. AI is a marker for arterial stiffness, an independent predictor of cardiovascular and cerebrovascular diseases, and a consequence of endothelial function impairment [31,32]. In our study, AI decreased after the WW+Tyr intervention. Thus, our data agree with previous reports concerning the benefits of polyphenol-rich VOO on endothelial function in humans [33].

As expected, HDL-c increased after both treatments in which moderate alcohol consumption was involved [34]. HDL protects endothelial function by preventing LDL oxidation [35]. Within the frame of the EUROLIVE Study we reported 1) a dose-dependent increase of plasma HDL-c, a decrease in LDL oxidation [2], and an improvement of HDL functionality [36] with the phenolic content (mainly OHTyr and Tyr) of the olive oil administered and 2) the capacity of the OHTyr biological metabolites to bind to the HDL lipoprotein in a dose-dependent manner with the OHTyr content of the olive oil administered [36]. Together these impacts enhance HDL fluidity [36] and protect HDL from oxidation, thus rendering the particle fully functional [35]. In agreement with our previous data [2], in the present study HDL-c increased in a dose-dependent manner with the Tyr and OHTyr content of the beverage administered.

We observed improvements in Hcy and ATIII after the WW+Tyr intervention. Hcy, a risk factor for CVD and deep vein thrombosis, promotes endothelial dysfunction

through several mechanisms including NF□B activation [28]. Here we showed an increase in Hcy levels after the WW treatment, consistent with increases in Hcy levels after moderate alcohol and red/white wine consumption as previously described [37]. The addition of Tyr to WW abolished this increase in Hcy. Hcy also has prothrombotic effects [38]. Cerebrovascular disease patients with high urinary Hcy/creatinine ratio have been reported to have lower concentrations of ATIII [39]. Accordingly, we observed a concomitant decrease of ATIII as well as Hcy. ATIII, a pleiotropic molecule with anti-coagulation and anti-inflammation effects, contributes to an adequate blood flow [40]. Flow perturbation mediates neutrophil recruitment and potentiates endothelial injury [41]. The anti-inflammatory properties of ATIII include the inhibition of neutrophil recruitment and NF□B pathways [42]. NF□B activation promotes that of the inflammatory mediator iNOS [43]. Likewise, iNOS inhibition improves endothelium-dependent vasodilatation in aged rats [44]. Hcy disrupts the angiogenesis of the microvascular endothelium through an increase of *iNOS* expression [45]. The anti-inflammatory capacity of ATIII includes *iNOS* expression inhibition [46]. In agreement with the changes observed in Hcy and ATIII in our study, the increase in *iNOS* after WW was abolished after WW+Tyr treatment. In experimental models, Tyr, OHTyr, and their biological metabolites inhibit *iNOS* expression [47]. *iNOS* inhibition attenuates ET1 levels [48]. In agreement with this, in our study, post-treatment ET1 concentrations after WW+Tyr decreased versus the WW ones, particularly in the NA group.

As *iNOS*, soluble *CD40L* also induces endothelial dysfunction [49]. We have previously described a decrease in *CD40L* expression *in vivo* in healthy volunteers which was associated with the high phenolic content (mainly Tyr and OHTyr) of VOO [16]. Consistent with this, in the present study, WW+Tyr reduced *CD40L* expression. Similar decreases occurred after wine and cocoa flavonoid intake [50,51]. We observed decreases

in *p65 (RELA)* and *CFH* after WW+Tyr. p65 (*RELA*) is a transcription factor belonging to the NFκB family and its inactivation, like that of *CFH,* improves the inflammatory status [52]. *CFH* competes with C1q for binding to target surfaces [53], and C1q exerts inhibitory effects on pro-inflammatory gene expression by reducing NF□B mediated transcription through a p65 decreased activation[54]. Thus, in our study we observed concomitant reductions in *p65* and *CFH* expressions after WW+Tyr intervention. Red wine polyphenol extracts decrease both *NFkB (p65*) and *iNOS* expressions [55], and inhibition of *p65* translocation by olive tree (*Olea Europea* L) methanolic extracts has been recently reported [56]. Tyr-4-sulfate prevents TNF-α-induced NF-κB signaling in endothelial cells by reducing p65 phosphorylation [57]. The WW+Tyr treatment eliminated the increase observed in the expression of *p65/RELA, VEGFA, eNOS*, and *CFH* after WW. At present there are no data from human *in vivo* studies concerning the effect of alcohol and wine on the expression of these genes. VEGF induces proangiogenic changes such as vessel dilatation and increased vascular permeability predominantly via *eNOS* [58], and *VEGF* inhibition decreases *CFH* in the eye and kidney [59].

Our findings confirm the involvement of *CYP2A6* and *CYP2D6* enzymes in the endogenous formation of OHTyr from Tyr in vivo in humans. The fact that individuals with low PAS have higher Tyr recoveries, and those with normal activity showed a higher OHTyr/Tyr ratio at the end of the WW+Tyr intervention confirms a role for genetic modulation of these enzymes in Tyr metabolism to OHTyr. Differences in the observed health effects following interventions, between LA and NA metabolizers, allowed the identification of which phenolic compound, Tyr or OHTyr, was preferentially involved in the changes observed. The fact that the increase in RHI, and the decrease in AI, after WW+Tyr occurred preferentially in the LA group, where Tyr recovery is high and OHTyr/Tyr ratio is low, indicates Tyr as the likely main responsible phenol for RHI and

AI improvements. Conversely, the fact that the decrease in Hcy and the increases in ATIII and HDL-c after the WW+Tyr treatment occurred particularly in the NA group, where OHTyr and OHTyr/Tyr recovery ratios are high, provides evidence that OHTyr is the main phenol involved in these improvements. Similarly, decreases in *CD40L* occurred particularly in the NA group suggesting OHTyr as the key phenol involved.

Our study has strengths and limitations. One strength is the crossover design which minimizes the role of possible confounding variables and between-subject variation. The similarity of matrices between WW and WW+Tyr treatments permitted us to assess the role of the exogenous Tyr administered and its bioconversion into OHTyr. One limitation is the sample size, particularly for women. The length of the study (6 months) and the restricted inclusion criteria may have decreased study recruitment for both genders. Tyr and alcohol doses were higher in men than in women may contribute to the higher responsiveness to interventions in men. Although Tyr and OHTyr effects are influenced by PAS, not all of the variability in OHTyr and Tyr recoveries could be explained by variants assessed for *CYP2A6* and *CYP2D6* and used in the PAS. Besides, additional untested variants, and potential alternative enzymes contributing to variation, enzyme expression can be affected by further factors such as drugs, diet or hormones. We were unable to assess treatment interference due to dietary components or medication. The fact that participants were CVD risk individuals limits the extrapolation of the results to the general population. Whether additional or different effects would have been observed over longer periods is unknown, nevertheless, greater intervention periods could have compromised participants' compliance.

In conclusion, ours is the first study to demonstrate the endogenous conversion of Tyr into OHTyr occurs in humans in vivo. This describes a new mechanism that could explain, in part, the potential benefits associated with moderate wine consumption and of

other Tyr rich components of our diet. Moreover, we have shown that both Tyr and its partial biotransformation to OHTyr promoted cardiovascular health-related benefits in humans after the intake of Tyr, in the context of real-life diet pattern with wine intake with meals. The study design allowed the health effects of individual phenols to be singled out from the dietary matrix in which they are built-in. Tyr bioconversion to OHTyr is modified by *CYP2A6*/*CYP2D6* genetic polymorphisms, and therefore some individuals may benefit more than others from the biological activities of these antioxidants. The results obtained in the present work encourage further study of Tyr rich foods and/or Tyr supplements with other foods or beverages for added enhancement of the healthy bioactivities in vivo.

Authors' contributions to the manuscript:

The manuscript was written by RTF, AB, MCI, RFT, and MF.

The research was conducted by AB, JM, NS, MG, JRM, CV, DM, FB, RFT and CPM.

KL analyzed the data.

MIC provided essential help with the project conception.

RTF designed the research.

All authors read and approved the manuscript

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Conflict of interest

RFT has been consulted by Quinn Emanuel, Ethismos and Apotex on unrelated topics. The other authors declare no other conflicts of interest.

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